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Decrease in fluorescence lifetime by glycation of collagen and its application in determining advanced glycation end-products in human dentin

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Abstract: Advanced Glycation End-products (AGEs) are produced by the Maillard reaction, which causes cross-linking of collagen and results in changes in the mechanical properties of collagen tissues. Several types of AGE fluoresce, and measurement of this fluorescence is effective for determining the presence of AGEs. Because fluorescence intensity by steady-state fluorometry is affected by sample surface condition and light source, we focused on fluorescence lifetime measurement (FLM). We found that fluorescence lifetime of collagen gel decreased with glycation progress. *In vivo* application of FLM for determination of AGEs was confirmed in human dentin.

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1. Introduction

Glycation via the Maillard reaction is the non-enzymatic reaction between protein and reducing sugars such as glucose and ribose, resulting in the formation of Advanced Glycation End-products (AGEs) [1]. Because the glycation of collagen progresses under physiological conditions in living organisms, glycation is one of the most important processes in human aging. Several types of AGE bind to collagen and act as cross-links between collagen fibrils, resulting in changes in mechanical characteristics of collagen-rich tissues.

In a previous report, we confirmed the accumulation of AGEs in human dentin with respect to age by mechanical indentation measurement of demineralized tooth sections and immunohistochemical staining [2], and confirmed the following phenomena; aged dentin was stiffer than the young dentin, and the stiffness of young dentin increased by glycation when tooth sections were incubated in ribose solution.

AGE is the collective term for Maillard reaction products, and several types of AGE emit autofluorescence under excitation with UV light. Among the fluorescent AGEs, pentosidine is a main component of the fluorophores [3–6]. As the formation of non-fluorescent AGEs, such as carboxymethyl lysine (CML), is largely synchronized with fluorescent AGEs [7], fluorometry is a valuable tool for detecting AGEs in biological tissue. However, fluorescence intensity from steady-state fluorometry changes depending on sample surface characteristics and/or fluctuations of the light source when examining *in vivo* tissue. This implies resultant fluorescence intensity does not necessarily correspond to AGE levels during *in vivo* measurement.

An alternative method to steady-state fluorometry is time-resolved fluorometry, namely fluorescence lifetime measurement (FLM), and this approach is widely used together with steady-state fluorometry in clinical settings [8–10]. However, there have been few reports on the application of FLM to examination of AGEs. Although we previously reported that fluorescence lifetime of human dentin decreased with increasing age [11], that report lacked discussion with respect to the AGEs.

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In the present study, we prepared collagen gels incubated with ribose and measured changes in fluorescence lifetime of the gels in order to examine the applicability of FLM to detect AGEs. The formation of AGEs in the gel was confirmed by western blotting. We then extended FLM to visualize the AGE distribution in human dentin. Based on the resultant profile, we discuss dental physiology with respect to the accumulation of AGEs in human dentin.

2. Materials and methods

2.1 Sample preparation

2.1.1 Collagen gel

Six pairs of gel-mounted dishes with different incubation times were prepared. Type I collagen solution for cell culture (3.0mg/ml, Type I-P; Nitta Gelatin, Osaka, Japan) and ten-fold concentrated phosphate buffered saline (PBS) were mixed on ice at a ratio of 9:1. Then, 200 μL of this solution was placed onto a glass-base dish for cell culture, and was incubated for 30 min at 37°C to form a gel. In vitro glycation of the gel was performed by adding 1 mL of 0.1 M ribose in cacodylate buffer (pH 7.4) onto a dish, while the same buffer without ribose was placed into another dish as a control (first pair dish). This pair was incubated at 37°C until examination.

Other pairs were prepared using the same procedure every week. Six weeks after start of the first glycation reaction, all gels were rinsed with PBS twice to remove residual ribose for measurement.

2.1.2 Human teeth

Caries-free third molars of young and elderly patients (young, 22-26 years; elderly, 68-76 years), extracted as a part of routine treatment at Osaka University Dental Hospital, were prepared for the experiment. Teeth were collected with patient consent and were preserved in Hank's balanced salt solution (HBSS; pH 7.4) at 4°C until the experiment. Experimental protocols were approved by the Ethics Committees of the Faculty of Dentistry, Osaka University.

Samples were sliced parallel to the tooth axis with a diamond wheel saw under water in order to obtain 1-mm slices. Sections were demineralized for four weeks with 10% ethylenediaminetetraacetic acid (EDTA) at room temperature. For *in vitro* glycation of a tooth sample, demineralized sections were incubated in 0.1 M ribose solution in HBSS at 37°C for 6 weeks, similarly to a previous report [2].

2.2 Fluorometry

2.2.1. Steady-state fluorometry

In situ fluorescence of the collagen gels was photographed with a conventional digital camera under illumination at 365 nm from a mercury lamp. Fluorescence emission spectra of the gels were measured with an ordinary fluorescence spectrophotometer (RF-5300; Shimadzu, Kyoto, Japan). For measurements, each gel was suspended in 1 mL PBS/0.1%SDS and sonicated with an ultrasonicator (VP-050; Taitec, Saitama, Japan), and the suspension was dialyzed against PBS. Scattering of excitation light in the gel suspension causes spectral distortion of observed fluorescence spectrum when the excitation wavelength is near the emission wavelength. Therefore, the excitation wavelength in this experiment was set at 350 nm, while that of FLM was 375 nm, and a UV-cut filter (cut-off wavelength: 405 nm) was placed in front of an emission monochromator to prevent spectral distortion.

2.2.2. Time-resolved fluorometry

The scheme for time-resolved fluorescence microscopy is shown in Fig. 1. An epi-illumination fluorescence microscope (Fluorophoto; Nikon, Tokyo, Japan) equipped with a motor driven

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X-Y stage was used. As a pulsed excitation light source, a picosecond diode laser (Hamamatsu PLP-10, emission wavelength 375 nm, pulse width 70 ps, repetition frequency 5 MHz; Hamamatsu Photonics, Shizuoka, Japan],) was employed. Time-resolved fluorescence from the specimen was detected with a fast photomultiplier (PMT; R-1635P; Hamamatsu) in photon counting mode, and the resultant photoelectrons were analyzed using a time-correlated single photon counting module (TCSPC; SPC-630, electrical time resolution 8 ps; Becker&Hickl, Berlin, Germany,) consisting of a fast amplifier, a reverse-mode time-to-amplitude converter and a pulse height analyzer. To minimize unwanted distortion of the resultant fluorescence decay curve due to multiphoton pile-up, the counting rate (detection frequency/excitation frequency) was set to within 2% by adjusting the variable ND filter.

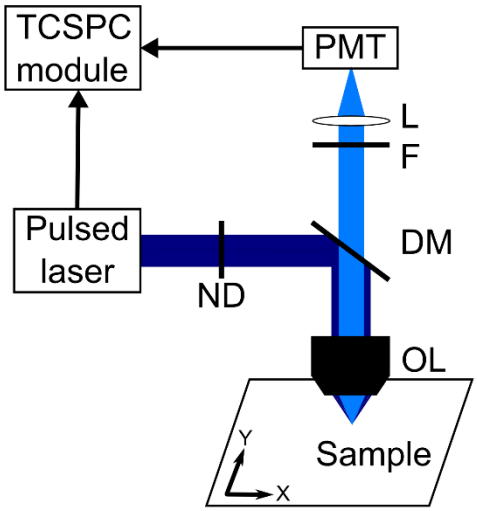

Fig. 1. Schematic diagram of time-resolved fluorescence microscope. ND: neutral density filter; DM: dichroic mirror; F: low pass filter; L: lens.

For FLM imaging of dentinal collagen with high magnification, a confocal laser microscope (C1, Nikon, Japan) equipped with the same pulsed laser described above was employed.

The observed fluorescence decay curve F(t) consisted of multiple exponential composite curves as Eq. (1), where Ai is the weight of the composite curve with a lifetime of τ_i .

$$F(t) = \sum A_i \exp\left(-\frac{t}{\tau_i}\right) \tag{1}$$

where, $F(t)$ is decomposed to the composite curves using a curve fitting program (Igor, WaveMetrics). From the resultant composite curves, "average lifetime" τ was calculated by Eq. (2) according to Lakowicz [12].

$$\tau = \frac{\sum A_i \tau_i}{\sum A_i} \tag{2}$$

2.3. AGE determination by western blotting

Glycated collagen gels were crushed with a pipette tip and were dissolved in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, followed by boiling at 94°C. Total protein concentration was determined using a Protein Assey Kit (Bio-Rad Protein Assey Kit; Bio-Rad, CA) based on the Bradford method [13].

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Demineralized human dentin was homogenized with a bead crusher (μT-01; Taitec) and an ultrasonicator, and was further solubilized with 3M HCl for 24 h at 37°C [14,15] after finishing fluorescence measurements. Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) analyses of collagen samples were performed on a gradient separating gel of 4-15% (Mini-PROTEAN TGX gels; Bio-Rad) according to Laemmli's method [16]. In this analysis, 5 μg of crude sample was loaded in each lane. Following electrophoresis, samples were stained with 2D-Silver Stain Reagent II (Cosmo Bio, Tokyo, Japan), and were transferred electrophoretically to PolyVinylidene DiFluoride (PVDF; Bio-Rad) membrane by using semi-dry blotter (Trans-Blot® SD; Bio-Rad) with a Tris/CAPS buffer system for large molecular proteins [17]. PVDF membranes were treated with blocking agent (Ez Block Chemi, ATTO Corp., Tokyo, Japan), and were then immunostained with primary antibody (1: anti-CML, clone no. 6D12; 2: anti-pentosidine, clone no. PEN12; Transgenic Inc., Kumamoto, Japan) for 1 h and with secondary antibody (Abcam, Cambridge, UK) for 1 h, successively. Both primary and secondary antibodies were diluted to 1:5000. Immunobound levels of proteins were visualized by chemiluminescence for pentosidine and tetramethylbenzemidine (TMB) staining for CML with horseradish peroxidase (Ez West Lumi plus and Ez West Blue; ATTO Corp.). Chemiluminescence was determined with a luminescent imager (Versa Doc; Bio-Rad). For human dentin samples, the modified Kivirikko's method was applied for quantification of hydroxyproline to calibrate the amount of collagen in this assay [18,19].

2.4 Immunohistochemical staining

Demineralized dentin blocks were embedded in paraffin blocks after graded-ethanol dehydration, and were then sliced into 4-mm sections using a microtome (Leica Microsystems GmbH, Wetzlar, Germany). Primary antibody that recognizes AGEs (anti-CML, Clone No. 6D12; Transgenic Inc.) was then applied, and fluorescence from the stained specimen was measured under an excitation wavelength of 588 nm with a confocal laser microscope.

3. Results

3.1. Type I collagen gel

3.1.1. Fluorometry

Figure 2 shows fluorescence images of the collagen gel with and without ribose under mercury lamp illumination. As another control, fluorescence images of collagen-free dishes that were filled with buffer (A) and ribose solution (B) are shown. Collagen emitted blue light after incubation in ribose solution, and its intensity increased with incubation time (C). However, fluorescence of the collagen did not increase appreciably when incubated without ribose.

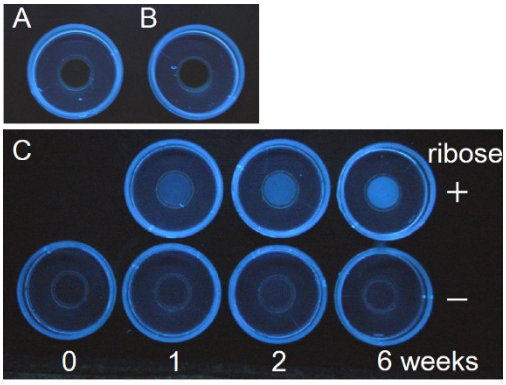

Fig. 2. Photographs of dishes under illumination with a mercury lamp (365 nm). Upper panel: collagen-free dishes filled with PBS (A) and ribose solution (B). Lower panel: collagen-mounted dishes incubated with and without ribose solution (C).

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In order to clarify the fluorescence properties, fluorescence emission spectra of the collagen gels were measured with the fluorometer. The resultant spectra are shown in Fig. 3, where the control corresponds to the dish for "week 0" in Fig. 2. As shown in the figure, fluorescence intensity increased with incubation time. There were marked differences in fluorescence spectra between the control gel and the 6-week gel; the control showed a trapezoidal shape, while the 6-week gel showed a triangular shape. Such differences in spectral shape indicate the formation of fluorescent components due to reaction with ribose.

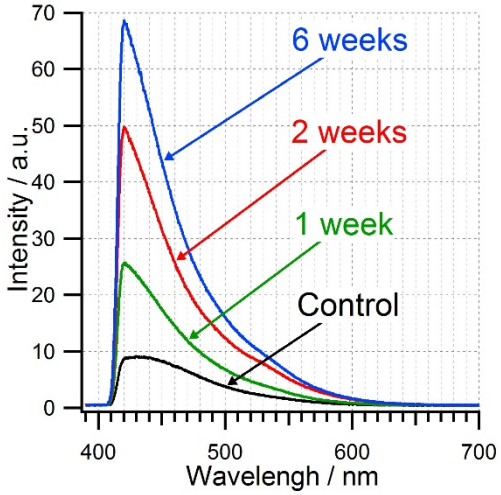

Fig. 3. Fluorescence emission spectra of collagen gel incubated with ribose for 0, 1, 2 and 6 weeks.

Time-resolved fluorescence profiles of the ribose-treated gels indicated that the fluorescence intensity decayed faster with increasing incubation time, as shown in Fig. 4. We extended incubation time to 9 weeks to examine whether fluorescence decay would further increase, and the resultant curve indicated further reaction between residual collagen and ribose.

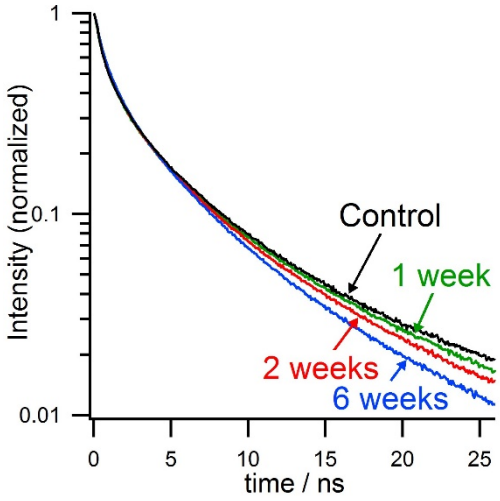

Fig. 4. Fluorescence decay curves of collagen gel incubated with ribose for 0-6 weeks.

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We assumed that the observed fluorescence decay curve $F(t)$ was composed of three exponential decay curves, as shown in Eq. (3), in order to simplify numerical analysis, although there are more possible decay composites.

$$F(t) = A_1 \exp\left(-\frac{t}{\tau_1}\right) + A_2 \exp\left(-\frac{t}{\tau_2}\right) + A_3 \exp\left(-\frac{t}{\tau_3}\right) \tag{3}$$

where, the resultant values for τ_1, τ_2 and τ_3 were 0.56 ± 0.04 ns, 2.8 ± 0.2 ns and 10.5 ± 1.5 ns, respectively. Based on these values, the average fluorescence lifetimes, τ_A and τ_B, were calculated using Eq. (4).

$$\tau_A = \frac{A_1\tau_1 + A_2\tau_2}{A_1 + A_2}, \qquad \tau_B = \frac{A_1\tau_1 + A_2\tau_2 + A_3\tau_3}{A_1 + A_2 + A_3} \tag{4}$$

Figure 5 shows the change in average fluorescence lifetimes with respect to incubation time in the ribose solution. With increases in incubation time, the value of τ_B decreased, while that of τ_A was around 1.6 ns.

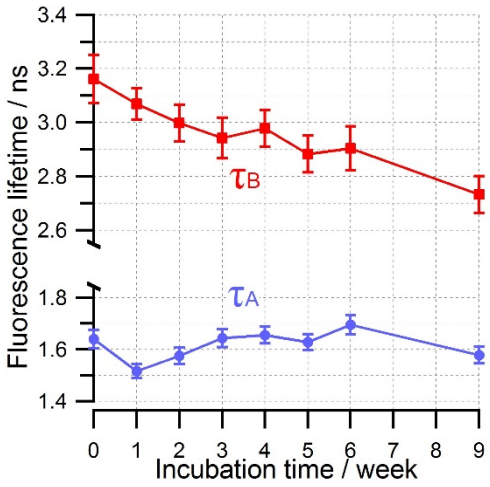

Fig. 5. Changes in average fluorescence lifetimes of collagen gel with respect to incubation time in ribose solution. τ_A: average lifetime of fast and second decay components; τ_B: average lifetime of three decay components.

3.1.2 Electrophoresis and western blotting

In order to confirm that fluorophore originates in AGEs, SDS-PAGE and western blotting analysis were applied to glycated collagen gel samples using anti-AGE antibodies (Fig. 6). SDS-PAGE of crude collagen gel samples (lane numbering according to incubation period in weeks) showed almost the same amount of around 250 kDa protein weight, except for weeks 0 and 1 (A). On the other hand, western blotting analysis with anti-AGE antibody showed higher binding to 250-kDa proteins after 2 weeks (B, C), while the week 0 and week 1 samples had little or no binding, despite large amounts of protein (A). Figure 6(B) showed that the total amount of CML became saturated within 2 weeks, while Fig. 6(C) showed the amount of pentosidine-bound antibody gradually increased with incubation period.

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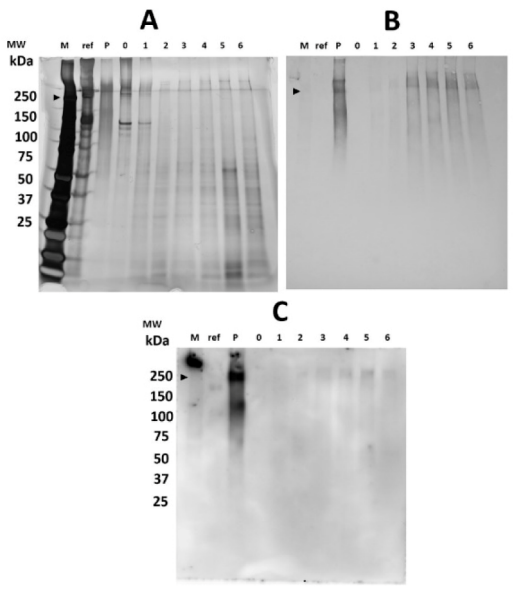

Fig. 6. SDS-PAGE (A) and western blotting of collagen gel samples (Anti-CML antibody (B) and anti-pentosidine antibody (C)). 0 to 6: Reaction period (weeks); M: Protein standards; ref: Calf skin collagen as reference; P: Calf skin collagen glycated with D-ribose as a positive control. (black arrowheads: 250 kDa).

3.2 Human dentin

3.2.1 Fluorometory

Figure 7 shows typical fluorescence decay curves of demineralized dentin measured in the same sample four times over six weeks (at 0, 1, 3 and 6 weeks after sample preparation without ribose) in order to examine fluorometric stability of the tooth sample. As there were no appreciable differences between the resultant decay curves, we conclude the dentinal collagen is sufficiently stable to examine by FLM, even when long periods are required for measurement. One fluorescence decay curve for glycated collagen gel was superimposed in Fig. 7 to examine the decay profile of dentin. As fluorescence decay of the dentin is longer than that of collagen, we assumed that the observed decay curve of the dentin consisted of two exponential components to simplify the curve fitting procedure; one is due to AGEs and the other is due to residual tissue fluorophore whose fluorescence lifetimes and component weights are τ_a, A_a and τ_r, A_r, respectively. The average fluorescence lifetime of dentin, τ_D, was thus calculated as:

$$\tau_D = \frac{A_a\tau_a + A_r\tau_r}{A_a + A_r} \tag{5}$$

In the practical curve fitting, we fixed τ_a at 1.6 ns, which was obtained from the glycated collagen gel as τ_A, as noted above. Distribution of τ_D values on demineralized dentin sections from young and aged teeth is shown in Fig. 8, where the FLM images were obtained before and after incubation in ribose solution.

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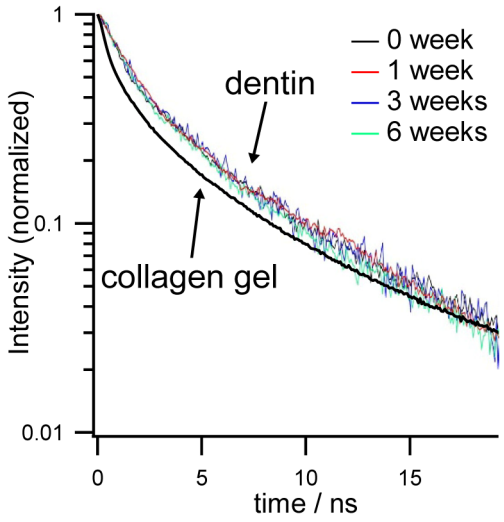

Fig. 7. Fluorescence decay curves of dentin tissue and collagen gel. Fluorescence decay curve of the same dentin incubated in buffer solution at 37°C without ribose was measured 4 times at 0, 1, 3 and 6 weeks after sample preparation. Collagen gel was incubated for 2 weeks in ribose solution.

As shown in this figure, fluorescence lifetime of the root portion was shorter than the crown in the aged dentin before incubation (Fig. 8(A), 8(B)). Fluorescence lifetime in young and aged dentin decreased after incubation with ribose, and the resultant lifetime imaging showed homogeneous distribution (Fig. 8(C), 8(D)).

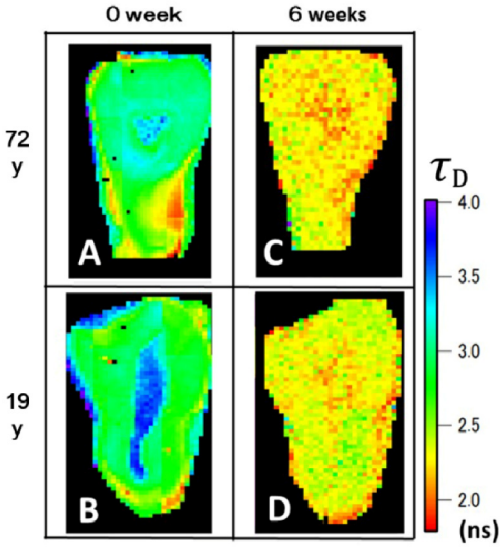

Fig. 8. Distribution of average fluorescence lifetime τ_D on demineralized dentin before and after incubation in ribose solution for 6 weeks. (A) and (B) show young and aged dentin before incubation, (C) and (D) show young and aged dentin after incubation.

Magnified images of the root portion are shown in Fig. 9, where both fluorescence intensity and lifetime were measured using confocal laser microscopy, as described above. There are round dark portions in the intensity image (Fig. 9(A), 9(B)), and these correspond to dentinal tubules whose surroundings show short lifetime area (Fig. 9(C), 9(D)). When the aged section

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was immunostained with anti-AGE antibody, the areas surrounding the dentinal tubules showed good fluorescence (Fig. 9(E)). This indicates reasonable consistency between fluorescence lifetime image and the presence of AGEs.

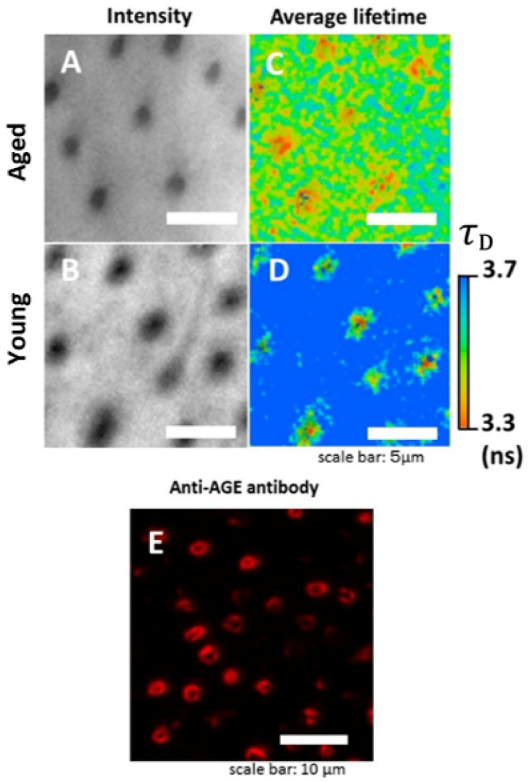

Fig. 9. Distribution of fluorescence intensity (A, B) and average fluorescence lifetime τ_D (C, D) of young and aged dentin sections. (E) Distribution of AGEs on fluorescent anti-AGE immunostaining. Scale bar: 10 μm.

3.2.2. Western blotting and immunohistochemical assay

Figure 10 shows the concentration of AGEs in human dentin, as examined by SDS-PAGE and western blotting. The amount of protein present in Silver-stained gel lanes was similar to that of glycated gel samples in western blotting lanes. We confirmed the presence of both CML (Fig. 10(B)) and pentosidine (Fig. 10(C)), and found their concentrations in aged dentin to be higher than those in young dentin.

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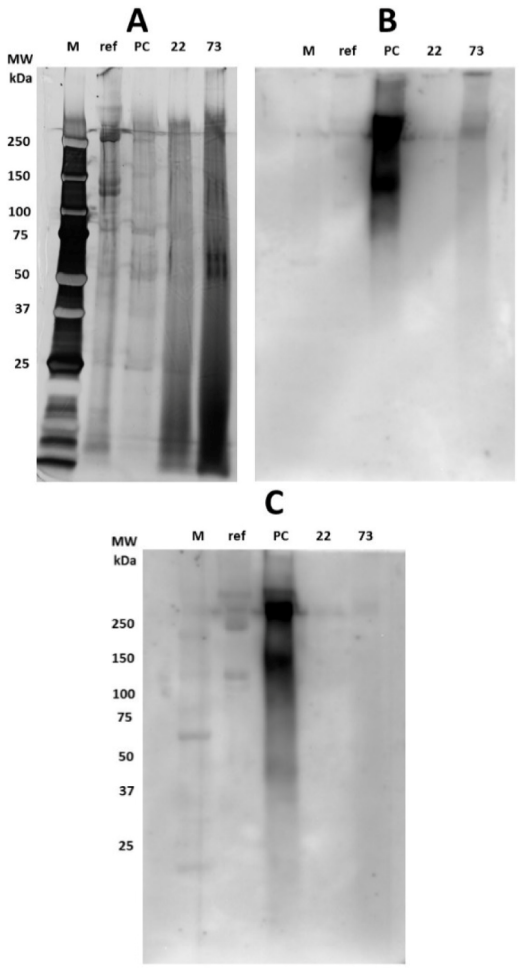

Fig. 10. SDS-PAGE (A) and western blotting (B, C) of human dentin collagen samples with anti-CML antibody (B) and anti-pentosidine antibody (C). M: Protein standards; ref: Calf skin collagen; PC: Calf skin collagen glycated with D-ribose as a positive control; 22: 22-year-old subject (molar tooth); 73: 73-year-old subject (molar tooth).

4. Discussion

Accumulation of AGEs can be detected by fluorescence measurement. Therefore, non-invasive diagnosis of diabetes is possible by applying fluorometry, and diabetic screening equipment such as the AGE reader (Diagnoptics Technologies B.V., Groningen, Netherlands) is now commercially available. The fluorometry method used for this purpose is the steady-state fluorometry. However, *in vivo* measurement using steady-state fluorometry is influenced by fluctuations in the excitation intensity and sample surface properties such as coloration and roughness, even when using it to measure hard tissue samples such as tooth sections. Observed fluorescence intensity in aged teeth was lower than in young teeth (Fig. 9(A), 9(B)) in the present experiment, although we reported that fluorescence intensity of the former was higher than that of the latter in previous experiments [11,20]. Such inconsistencies are caused by differences in sample surface condition and instrumentation between the present experiment and previous studies. One attractive technique that complements steady-state fluorometry is FLM. However, there have been few reports on the applicability of FLM to examination of AGEs *in vivo*. For example, Blackwell et al. used FLM on the human skin for diagnosis of

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diabetes, and showed non-significant difference between the fluorescence lifetimes of nondiabetic and diabetic subjects [21]. Thus, the relationship between fluorescence lifetime and degree of glycation remains ambiguous. We should determine the relationship between formation of AGEs and fluorescence decay profiles via *in vitro* measurement using an adequate model before applying FLM to *in vivo* measurements.

Type I collagen is predominant in the human body; therefore, we focused on glycation of type I collagen in our *in vitro* examination. As the reducibility of ribose is stronger than that of glucose [22], collagen was incubated in ribose solution rather than glucose solution, which corresponds to blood sugar, for *in vitro* glycation in order to shorten the incubation time.

As shown in Figs. 3 and 4, fluorescence intensity and fluorescence decay time of collagen increased and decreased, respectively, by glycation. As the fluorescence decay curve was not a single exponential decay, but showed multiple exponential decays, we expressed the decay speed by taking the average fluorescence lifetime as Eq. (2), assuming that the observed decay curve consists of 3 exponential components, as shown in Eq. (3). As the resultant value of τ_B decreased with increasing incubation time, while that of τ_A remained around 1.6 ns, we can suppose that τ_A is a characteristic value of the generated products. The value for τ_B, which is the average fluorescence lifetime of the collagen gel, reflects the concentration ratio of the generated products to that of residual collagen whose fluorescence lifetime corresponds to τ_3. As pentosidine is a main component of fluorescent AGEs [3–6] and CML is a non-fluorescent AGE, the observed fluorescence in the present experiment may be derived from pentosidine. To confirm this, pentosidine and CML in the glycated collagen gel was assayed electrophoretically. The results shown in Fig. 6 indicate the presence of both pentosidine and CML in the collagen gel. Such simultaneous formation of fluorescent and non-fluorescent AGEs is consistent with the report of Meerwaldt *et al*. [7]. Based on these analyses, we conclude that the decay curve from FLM of glycated collagen reflects the formation of AGEs.

More interestingly, we found that the possible fluorescence lifetime that characterizes pentosidine is 1.6 ns based on the curve fitting, although FLM of purified pentosidine is essential to provide credence and relevance of τ_A. However, we have not succeeded in extraction of sufficient amount of pentosidine for FLM from collagen gel or dentin in our current experiment, because pentosidine molecule is strongly bound to the collagen molecule.

In order to evaluate the applicability of FLM to *in vivo* determination of AGEs, imaging of the average fluorescence lifetime in human dentin was compared with the results of our previous report [2]. In the present experiment, the presence of AGEs in the dentin was confirmed by biochemical assay, and average fluorescence lifetime value was determined by assuming that the resultant fluorescence decay curve consists of two components conventionally, i.e., due to pentosidine and residual fluorophores in dentin tissue. In a previous report [2], the distribution of cross-linking AGEs in teeth was determined by mechanical indentation test for demineralized tooth sections under the expectation that cross-linking mechanically reinforces the dentinal collagen. That report showed that: (1) concentration of AGEs is higher in aged dentin than in young dentin; (2) concentration of AGEs in the root portion is higher than that in the coronal portion; (3) concentration of the AGEs increases when incubated in ribose solution; and (4) AGEs are accumulated around dentinal tubules. The resultant FLM imaging reflects the presence of AGEs and is consistent with the results described above in (1)-(4).

5. Conclusion

We conclude that FLM is able to provide useful information for the *in vivo* assay of AGEs in collagen, although it is necessary to further quantify the relationship between fluorescence lifetime values and degree of AGE formation. In clinical settings, it is currently difficult to define the boundary between sound dentin and carious dentin. However using the FLM imaging method, we can easily distinguish between the two, as dentinal caries contain pentosidine and CML [22–25]. Thus, this technique may have other clinical applications in the

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field of dentistry. In addition, steady-state fluorometry and FLM can be applied to the non-invasive diagnosis of diabetes.

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